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Freeze tolerance conferred by transgenic expression of late embryogenesis abundant protein in insect cells

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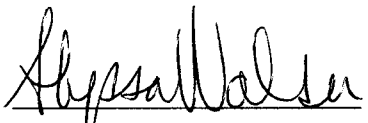

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Freeze tolerance conferred by transgenic expression of Late

Embryogenesis Abundant Protein in insect cells

(TITLE)

BY

Alyssa N. Walser

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IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
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Freeze Tolerance Conferred by Transgenic Expression of Late Embryogenesis Abundant Protein in Insect Cells

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A Thesis Presented to the Faculty of the Graduate School of Eastern Illinois University in Partial Fulfillment of the Requirements for the Degree of Master of Science of Natural Sciences (Research)

2013

Abstract

Anhydrobiotic organisms such as the brine shrimp *Artemia franciscana* have developed strategies to survive and thrive under conditions of limited water availability. One strategy is the accumulation of late embryogenesis abundant (LEA) proteins. Cells of *Drosophila melanogaster* (Kc167), which had previously been genetically modified to express a group 1 LEA protein (LEA1.3) found in *A. franciscana,* were used to test the impact of water stress due to freezing to -80°C. Two different freezing devices were used to determine the impact of the freezing protocol on the viability of cells after freezing and thawing. The first method used a 12-well microplate insulated in a novel passive freezing device produced by BioCision (Larkspur, CA) and the second method used traditional cryogenic vials. The membrane integrity of cells after freezing and thawing was compared between cells expressing LEA1.3 (Kc167-LEA1.3) and control cells that lack the transgenic protein (Kc167).

Furthermore, two different freezing solutions were employed. Efficiency of cryopreservation was compared between a standard freezing solution composed of cell culture medium (M3) and a novel choline-lactobionate based freezing buffer (ASFB), both supplemented with dimethyl sulfoxide (DMSO). There was a significantly higher amount of cells with intact membranes for Kc167-LEA1.3 cells than Kc167 wild type cells in both ASFB and M3 medium ($n = 12$, $P < 0.05$). This correlates with a higher rate of viability for cells expressing the *Af*LEA1.3 protein. Also, a significantly higher numbers of cells with intact membranes was found for both cell lines when frozen in 12-well plates as opposed to the freezing in 2 ml cryogenic vials ($n = 24$, $P < 0.05$). To ensure a steady freezing rate, two passive freezing devices were used. Plate based

freezing in multi-well plates may help to improve the experimental procedures utilized in cell storage and cell based bioassays. The expression of *Af*LEA1.3 protein and the use of a plate-based freezing device yields higher viability for cells exposed to extreme low temperatures.

Dedication

To My Husband

Acknowledgements

I first and foremost want to thank my advisor, Dr. Michael Menze. He provided me an opportunity to learn and grow beyond what I had expected, and was a constant source of support and guidance. He accepted me into his lab with open arms and helped to make my experience memorable. Without his positive attitude and willingness to be flexible, I would not have been able to accomplish even a fraction of what I have. I give thanks to Dr. Gary Bulla for his advisement and support on my committee, as well as his guidance throughout both my undergraduate and graduate work at Eastern Illinois University. I give thanks to Dr. Andrew Methven, without whom, I may never have pursued a graduate program in the sciences. He has always given me honest feedback, encouragement, and criticism, and I cannot thank him enough for answering all of my unending questions. I would also like to give thanks to Dr. Robert Fischer who pushed me to think bigger and go farther from the first day of my freshman year eight years ago.

Also, I would like to thank the Eastern Illinois Graduate School for allowing me the opportunity to continue my education by providing the Master of Science in Natural Sciences program. This program is a wonderful way for teachers who are also scientists not only to further their content knowledge, but also their desire to be better teachers by becoming better scientists. I thank all of my teachers and colleagues in the MSNS program. Without the constant encouragement of these wonderful people, I would not have found the motivation to keep going to accomplish this goal. Finally, I thank my husband for putting up with all the days and nights of reading, writing, and grading that come with a commitment like this. His continual support helped to pull me through the toughest moments and to celebrate the victories along the way.

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I. Introduction

A. Cryptobiosis

Life has adapted to the vast variety of environments available both on land and in water. Whether or not an organism can withstand any specific environmental condition has been molded by strong forces of natural selection. In the harshest of environments, organisms have two main options: to leave the biotope or to adapt strategies to cope with its conditions. One of the most frequent occurrences in animal species is to use migration to avoid harsh conditions by finding a more suitable habitat for a certain time and then returning when conditions improve (Dingle 1996). This behavior relieves the organism of intolerable conditions, but also has its own set of challenges. A great amount of energy is consumed by the journey itself, and there is also a measure of danger incurred by the path taken. The human impact on migratory patterns and habitats has added even more risk into the migration process (Lok et al. 2013).

If the distance or path to more tolerable conditions is too arduous, some animals find ways to adapt to the harsh conditions. In habitats that experience regular changes in conditions, some organisms favor a state of dormancy to survive the harsher times. Any state of resting is considered to be dormancy in spite of the season or time (Hand 1991; Hand & Hardewig 1996). During dormancy, organisms reduce their metabolic rates and slow or stop activities such as movement and development. The more activities are reduced, the longer the state of dormancy can last as resources are utilized at a lower rate. Sometimes, dormancy can even be prolonged if harsh conditions persist (Gremer et al. 2012).

Dormancy can be triggered either internally or externally. If dormancy is brought on by internal cues, such as hormonal changes, it is called diapause. Diapause is not directly dependent on environmental conditions and can continue even without harsh environmental conditions present (Bodine 1932; Womersley 1981; Cáceres 1997). If dormancy is triggered by external cues such as changes in season or environmental catastrophe, it is called quiescence (Clegg 2001). The animal can exit this state of dormancy only when environmental conditions improve. Quiescence can be caused by a variety of environmental cues and conditions including temperature change and desiccation (Yancey et al. 1982; Danks 1987; Hand 1998; Yancey 2005).

In organisms that experience a quiescent state, there are two distinct categories: organisms that simply slow their metabolic rate, or cryptobiots that have inestimable rates of metabolism (Keilin 1959). Cryptobiosis is exemplified by organisms which show no measureable signs of life, but can then return to a normal metabolic state when conditions improve (Crowe 1971). This state of almost complete arrest of life enables such organisms to utilize ecosystems most other organisms cannot. The type of environmental stress that triggers cryptobiosis can also help to define and categorize an organism. Cryptobiotic tolerance to water stress can be found in representatives of each kingdom and is known as anhydrobiosis (Giard 1894).

Water is an essential molecule to support life and can constitute up to 95% of the molecules found in invertebrate organisms (Edney 1977; Hadley 1994). Anhydrobiotic organisms can tolerate a loss of more than 77% body water, and, when rehydrated, can return to a normal state of development and reproductive success (Right 2001; Clegg 2005; Cornette & Kikawada 2011). There are a variety of organisms that display

anhydrobiotic properties including cysts of the brine shrimp *Artemia franciscana*, nematodes, rotifers, tardigrades, temperate and polar zone plant varieties, such as hardy wheat, and larvae of the insect *Polypedilum vanderplanki* (Crowe & Madin 1975; Glasheen & Hand 1988; Thomashow 1998; Treonis 2005; Cornette & Kikawada 2011; Guidetti et al. 2011; Wełnicz et al. 2011). While in the anhydrobiotic state, organisms can tolerate extreme conditions such as freezing, hypoxia, and gamma radiation while remaining viable (Watanabe et al. 2006; Jönsson 2007; Hayward et al. 2007).

When an organism is exposed to freezing conditions, intracellular water is lost and the cells experience extreme hyperosmotic stress. This state creates high concentrations of solutes within the cell which can lead to cell membrane damage (Elliot et al. 2008). All cells have the ability to withstand certain levels of water stress, but at high levels the increased solute concentrations can cause extreme interruption to cellular functions (Garner & Burg 1994). This increased stress on the cell may trigger apoptotic pathways or necrosis (Michea et al. 2002; Copp et al. 2005). Apoptosis is defined as programmed cell death that is synchronized, energy dependent, and due to specific cues that initiate and result in cell death (Elmore 2007). Near the end of apoptosis, cells package cytoplasmic contents into vesicles which can be cleared through phagocytosis (Jin & El Diery 2005). In contrast, necrosis results in cell death without apoptotic mechanisms and therefore does not result in the release of vesicles with intracellular contents (Elmore 2007). Water stress can also decrease transcription, which eventually causes the cell cycle to seize (Michea et al. 2000).

B. Initial Response to Hyperosmotic Conditions

When hyperosmotic conditions occur, cells experience a variety of unfavorable internal changes. When cells begin losing water, the intracellular volume decreases, which can lead to loss of membrane stability and even apoptosis if the condition persists (Bortner & Cidlowski 1998; Bortner et al. 2012). Cell shrinkage due to loss of internal water and therefore crowding of intracellular macromolecules induces transport of ions into the cell to provide short term relief from water loss (Al-Habori 2001; Wehner et al. 2003; Alfieri & Petronini 2007; Burg et al. 2007). This mechanism fails to provide support for organisms experiencing water loss due to freezing as all liquid water has become solid and lost the ability to flow freely into or out of the cell (Meryman 1956). For organisms experiencing hyperosmotic conditions at temperatures where water can remain a liquid, this increased intracellular solute concentration allows the cell to temporarily return to normal volume. The now increased concentrations of ions, such as chloride and sodium, can inhibit transcription and translation, leading to cell cycle interruption and when prolonged, cell death (Robbins et al. 1970; Greenberg 2004).

C. Late Embryogenesis Proteins (LEA)

Anhydrobiosis as a survival strategy to hyperosmotic conditions evolved independently in a wide variety of organisms from freeze tolerant seed variants to nematodes, insects, rotifers, roundworms, and cysts of the crustacean *Artemia franciscana*. Strategies employed by these organisms to tolerate hyperosmotic stress allow them to survive in conditions most organisms cannot. One strategy is the accumulation of intracellular proteins, such late embryogenesis abundant proteins (LEA)

(Liang & MacRae 1999; Jönsson & Schill 2007; Battaglia et al. 2008; Gusev et al. 2011; Hand et al. 2011).

In order for anhydrobiotic organisms to survive extreme freezing conditions and desiccation, all known anhydrobiotic organisms accumulate large amounts of structurally supportive proteins, such as LEA proteins, during hyperosmotic stress (Goyal et al. 2005). The presence of LEA proteins reduces the occurrence of proteins accumulating and clumping, and increases the energy required for proteins to irreversibly denature (Feder & Hofmann 1999; Goyal et al. 2005). LEA proteins are chaperone-like proteins that have significant impact on anhydrobiotic organisms. These proteins were first discovered in late embryonic stages of cotton seeds (*Gossypium hirsutum*) and resurrection plant seeds (*Craterostigma plantagineum*), which are both varieties that display significant desiccation tolerance (Dure et al. 1981; Galau & Hughes 1986; Bartels 2005). LEA proteins have also been found in cold tolerant plant variants of wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and rye (*Secale cereale*) where accumulation of chloroplastic LEA proteins allows the plants to develop freeze tolerance (NDong et al. 2002). In some freeze-tolerant plant cultivars there is accumulation of LEA proteins as freezing conditions are imposed, while there is a reduction in their concentration very shortly after deacclimation (Sutton et al. 1992).

Since their initial discovery in plants, LEA proteins have been found in a variety of organisms including bacteria, cyanobacteria, slime molds, and fungi as well as in animals representing four phyla including arthropods, nematodes, rotifers, and tardigrades (Close & Lammers 1993; Stacy & Aalen 1998; Sales et al. 2000; Battista et al. 2001; Katinka et al. 2001; Eichinger et al. 2005; Abba et al. 2006; Dure 2008; Hand et al. 2011). LEA

proteins have been found to be translated at a higher rates during hyperosmotic stress and directly before diapause of embryos in *A.franciscana* (Browne et al. 2004; Goyal et al. 2005; Kikawada et al. 2006; Hand et al. 2007; Menze et al. 2009; Menze & Hand 2009). At least 6 different LEA proteins exist in *A. franciscana* embryos (Hand et al. 2011).

LEA proteins are extremely hydrophilic macromolecules that assume enhanced α-helical secondary structure as water is removed from the cell (Chakrabortee et al. 2010; Tompa & Kovacs 2010). LEA proteins remain mainly as random coils during hydrated states most likely due to lack of hydrophobic residues. They confer tolerance to low water levels and chilling (Battaglia et al. 2008). The change in structural conformation prevents cell membrane rupture during water loss (Tolleter et al. 2007).

Desiccation tolerant animals have been found to express three of the six groups of LEA proteins known including group 1, group 3, and seed maturation like proteins (SMP-like) (Dure III et al. 1989; Hand et al. 2011; Wu et al. 2011). Group 1 and group 3 LEA proteins are better understood and more widely studied than SMP-like proteins in anhydrobiotic organisms (Tunnacliffe & Wise 2007; Hand et al. 2011). Genes of LEA proteins were found to have low temperature response elements in their promoters and many genes containing the respective promoter elements were induced by cold (Hundertmark & Hincha 2008).

Group 1 LEA proteins have a lower average molecular weight of 11.5 kDa, a more acidic pH, and a hydrophilic 20-amino-acid motif as compared to group 3 LEA proteins at 25.5 kDa, a more alkaline pH, and an 11-amino-acid motif (Dure III et al. 1989; Tunnacliffe & Wise 2007; Dure 2008). The specific function of LEA proteins is still unclear, but numerous studies have shown that these proteins help to stabilize proteins

and phospholipid membranes, therefore preventing protein clumping and maintaining membrane integrity (Hoekstra et al. 1997; Goyal et al. 2005; Chakrabortee et al. 2007; Pouchkina-Stantcheva et al. 2007; Tolleter et al. 2007; Tolleter et al. 2010; Tompa & Kovacs 2010; Furuki et al. 2012). Although many studies have been conducted and much knowledge has been gained about the structure and function of LEA proteins, their true function is still unknown.

D. Objectives of the Research

The main objective of this research was to employ hyperosmotic stress as induced by cell freezing to better understand the response of cells to this harsh environmental condition. I investigated the role of the protein *Af*LEA1.3 and varied the freezing regimens to develop new strategies to improve freezing tolerance of insect cell lines.

II. Materials and Methods

A. Kc167 and Kc167-LEA1.3 Cell Culture

Kc167 (*Drosophila melanogaster*) cells were obtained from the Drosophila Genomics Resource Center (DGRC, Bloomington, IN). Kc167 cells were cultured in 75-cm^2 cell culture flasks (Corning Corporation, Lowell, MA) containing standard cell culture medium (M3BPYE) consisting of Shields and Sang M3 insect medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Atlanta Biologics, Lawrenceville, GA), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 25 μg/ml amphotericin B (MP Biomedicals, Solon, OH), 0.5 g/l potassium bicarbonate, 1 g/l yeast extract (Sigma-Aldrich, St. Louis, MO) and 2.5 g/l trypticase peptone (BD Biosciences, San Jose, CA) adjusted to pH 6.6 with 1M KOH. Cells were grown to a density of 20 million cells/ml and subcultured to 1.5 million cells/ml bi-weekly.

In order to establish a proliferation curve for Kc167 and Kc167-LEA1.3 cells, samples of each cell line were exposed to the same procedures as used to test freeze tolerance without cooling of cells to -80°C. This allows measuring of any effects on cell viability due to processing so that cell death can be minimized and a sufficient number of live cells can be sampled after cryopreservation. A cell count was performed on a sample of each cell line. This was accomplished by removing 20 μl of suspended cells and adding 20 μl of trypan blue. Trypan blue is a large anionic dye, which can only penetrate the plasma membrane of cells that have lost membrane integrity. Dead or dying cells therefore stain blue while viable cells that maintain membrane integrity exclude the dye from the cytoplasm. After 30 seconds had passed, a cell count was taken using a droplet of the mixture under a counting grid of a hemocytometer. Cell counts were performed to

determine viability of cells based on the cells contained in each counting grid with intact membranes. With the cover slip in place, the volume of one large square is 1×10^{-4} ml subdivided into 16 subfields. In order to obtain the number of cells per milliliter, the number of cells counted in one large square is multiplied by 10,000 and by the dilution factor due to added trypan blue. This established a cell density for the subcultured samples to be used.

Cell samples were then centrifuged for 5 min at 175 *g* to remove the growth medium and resuspended in fresh growth medium to a volume that corresponded to a cell density of $3\text{-}5 \cdot 10^6\ ml^{-1}$. Cells were placed in a 12-well microplate with 3 ml of the cell solution in each well. Each plate contained six wells of Kc167 cells and six wells of Kc167-LEA1.3 cells. Final cell counts on each well were performed to confirm the prior calculated cell densities in each well. The microplate was then incubated for 24 h at 26.5°C.

After 24 h, cells were resuspended by pipetting and a cell count was performed on each well. The cell solution was then removed from each well and transferred into 10 mlsterile conical tubes along with a wash solution used to remove all cells from each well. These conical tubes were then centrifuged for 5 min at 175 *g* to pellet the cells and the supernatant was removed. Cells were resuspended in fresh growth medium and a final cell count was performed.

B. Response of Kc167 Wild Type and LEA1.3 Expressing Cells to Hyperosmotic Stress

1. Maximizing Cell Viability in Response to Freezing Buffer

Cell counts were taken from subcultured Kc167 cell samples to determine baseline cell densities. Samples were then centrifuged, the supernatant was discarded, and cells were resuspended in a variety of solutions. A control sample was treated with growth medium supplemented with 10% dimethyl sulfoxide to achieve a cell density of 5-8 • 10^6 ml^{-1}. Other samples were treated with a specially formulated freezing buffer (ASFB) composed of: 40 ml of 0.5 M K lactobionate per 250 ml buffer (lactobionate stock was composed of 35.83 g lactobionate dissolved in 200 ml H_2O and the pH was adjusted to ~7.0 with KOH), $MgCl*6H_2O$ (3 mM), Taurine (20 mM), KH_2PO_4 (10 mM), BSA (0.25g /250ml), sucrose (200 mM), pH 6.8 - 7.0 HEPES-KOH (20 mM). Each sample of freezing buffer was supplemented with varied quantities of dimethyl sulfoxide (DMSO), ranging from 0 to 10%, to determine the optimal DMSO concentration to maximize cell survival after one freeze-cycle. These cell samples were diluted to a cell density of 5-8 • 10^6 ml^{-1} using the freezing buffer described above. Once all cell samples were diluted in the proper solution, 500 µl of each sample was placed into three wells of a 12-well microplate and a final cell count was performed immediately before freezing.

Each microplate was placed on a novel passive freezing device (BioCision, Larkspur, CA) inside of an insulating chamber and placed at -80°C for 24 h. Microplates were then removed and each sample was allowed to thaw via the addition of 2.5 ml of growth medium at 37°C. Cells were then resuspended using a pipette and a cell count was taken for each well. The microplates were then incubated for 24 h at 26.5°C. Again, cells were resuspended using a pipette and a cell count was taken. The cell solution was then removed from each well and transferred into a 10 ml sterile microtube along with a wash solution used to remove all remaining cells from each well. These microtubes were then

centrifuged for 5 min at 175 *g* to pellet the cells and the supernatant was removed. Cells were resuspended in fresh growth medium and a final cell count was performed.

2. Freeze Tolerance EmployingTwo Different Devices

Cell counts were taken from subcultured Kc167 and Kc167-LEA1.3 cell samples to determine baseline cell densities. Each sample was centrifuged, the supernatant was discarded, and the cells were resuspended in either ASFB containing 10% dimethyl sulfoxide or growth medium supplemented with 10% dimethyl sulfoxide. These cell samples were diluted to a cell density of 5-12 • 10^6 ml^{-1} using the respective freezing solution. Within each trial, the cell densities of Kc176 and Kc167-LEA1.3 were kept within a range of ± 1 x 10^6 ml^{-1} for the medium-diluted and freezing buffer-diluted samples, respectively. Once all samples were diluted, 500 µl of each sample was placed into three wells of a 12-well microplate orinto three freezing vials. During each test, three samples each of Kc167 cells in ASFB, Kc167 cells in medium, *Af*LEA1.3 expressing cells in medium, and *Af*LEA1.3 expressing cells in ASFB were placed in each microplate. Each microplate was placed on the CoolCell freezing plate inside of an insulating chamber and each freezing vial inside of a freezing jar (BioCision, Larkspur, CA). Both cryogenic devices were placed at -80°C for 24 h.

Microplates and cryogenic vials were then removed and each sample was allowed to thaw via the addition of 2.5 ml of growth medium at 37°C. Cells were then resuspended and a cell count was taken. Samples in freezing vials were removed from the vials and transferred into wells of a 12-well microplate and incubated for 24 h at 26.5˚C. At the end of the incubation period cells were resuspended and a final cell count was taken.

III. Results

A. Kc167 and Kc167-LEA1.3 Cell Culture

To investigate the impact of the highly hydrophilic protein *Af*LEA1.3 on freeze tolerance of cells from *Drosophila melanogaster*, the impact of processing cells for cryopreservation was investigated. Both cell lines, Kc167-wild type and Kc167-*Af*LEA1.3, were exposed to all processing procedures with the exception of cooling to -80°C. Figure 1 shows the increase in percentage of cell number after incubation for 24 h. Only cells with intact membranes were included in the analysis. As expected, both cell lines roughly doubled during the 24 h incubation period (Figure 1, post incubation). However, cells that were exposed to the protocol employed to prepare for cryogenic storage showed a substantial reduction in cell proliferation ($n = 3$, $p < 0.05$, paired t-test)(Figure 1, post processing).There was no significant difference in membrane integrity for post incubation growth or post processing loss of between the two cell lines ($n = 3$, $p > 0.05$, paired t-test).

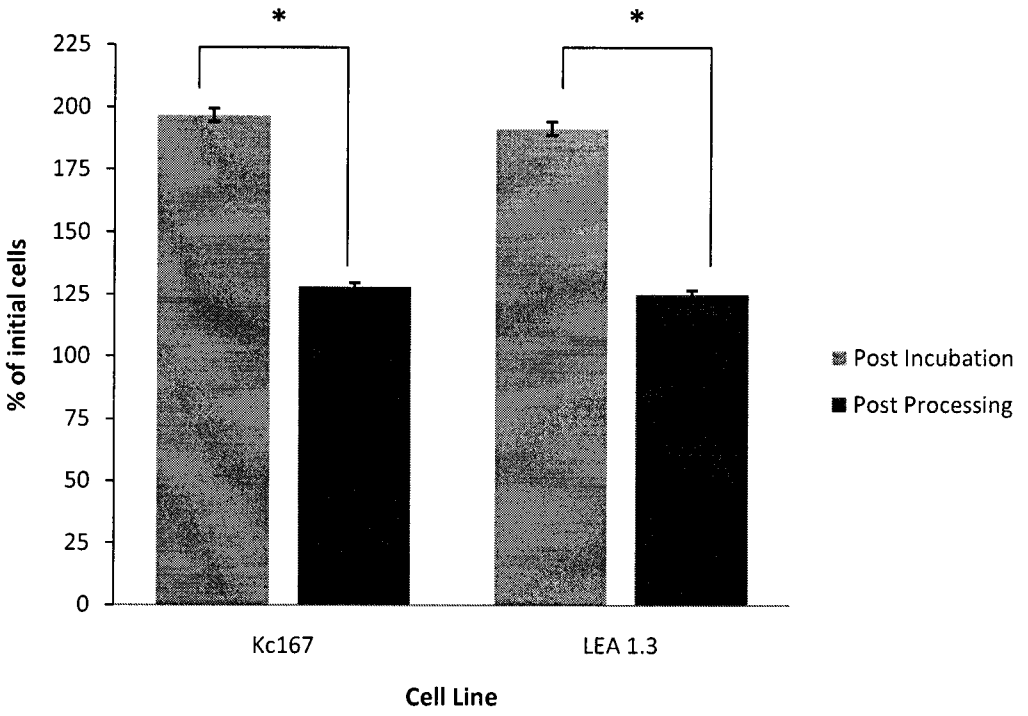

Figure 1.Response of *Drosophila melanogaster* Kc167 and Kc167-LEA1.3 cells to cell culturing protocols.

Counts of Kc167 and Kc167-LEA1.3 cells after 24 hours of incubation in cell culture medium and after cell processing procedures as described under Methods (II.A). The number of cells counted was expressed as a percentage of the number of cells initially plated.*statistically significant difference between paired means (n = 3, ±SE, $p < 0.05$).

B. Response of Kc167 Wild Type and LEA1.3 Expressing Cells to Hyperosmotic Stress

1. Impact of Dimethyl Sulfoxide on Cell Viability after Freezing

Kc167 cells were exposed to all experimental procedures including cooling of cells to -80°C in order to establish the most beneficial concentration of dimethyl sulfoxide (DMSO) in the freezing buffers employed. Figure 2 shows the percentage of cells with intact membranes after 24 h based on the number of viable cells before freezing to -80°C. There was a significant increase in the number of viable cells in both M3 medium and ASFB when 10% DMSO was added as the cryoprotectant as compared to all other DMSO concentrations tested ($n = 3$, $P < 0.05$). However, there was no significant difference in the number of viable cells frozen in the M3 medium or ASFB solutions using 10% DMSO ($n = 6$, $P > 0.05$).

In order to investigate the occurrence of delayed cell death after freezing, the membrane integrity of cells was measured 24 h after freezing and plating of cells to determine cell viability. Figure 3 shows the percentage of cells with intact membranes after 24 h of incubation at 26.5°C based on the amount of cells that initially survived freezing. There was a significant difference in the number of viable cells in ASFB when 10% DMSO was added as compared to all other concentrations tested ($n = 3$, $P < 0.05$). There was also a significant difference in the amount of cells with intact membranes between ASFB with 10% DMSO and M3 medium with 10% DMSO after 24 h, indicating a reduction in delayed apoptosisin cells frozen in ASFB ($n = 6$, $P < 0.05$).

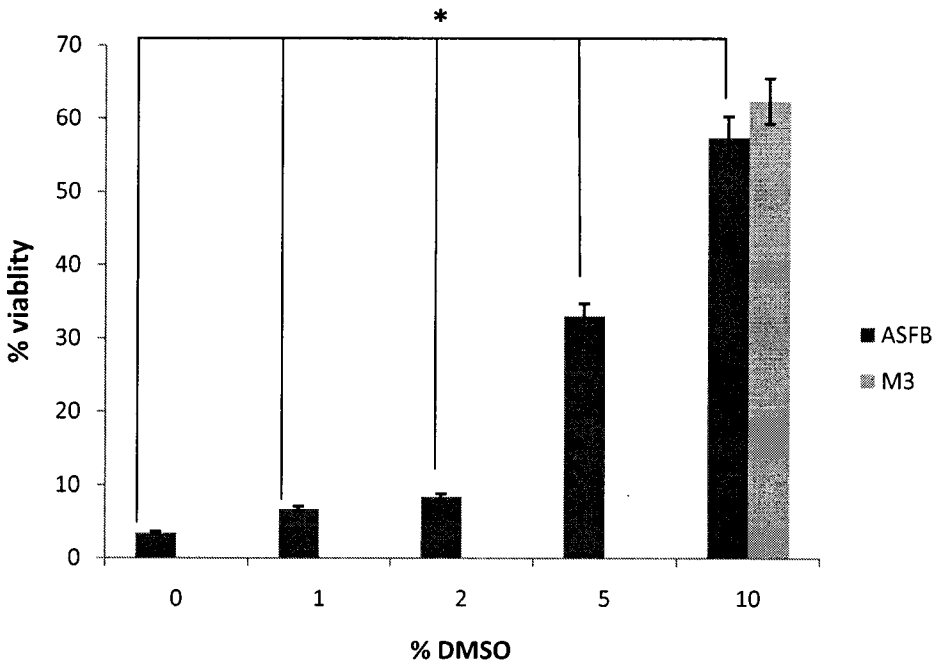

Figure 2.Response of *Drosophila melanogaster* Kc167 cells to cooling to -80°C.

Percentage of cells with intact membranes based on the initial number of viable cells before freezing.Numbers represent viability immediately post-freeze of Kc167 cells after cooling to -80°C in cell culture medium (M3) or ASFB with varying concentrations of dimethyl sulfoxide and slow thawing as described under Methods (II.B.1.). *statistically significant difference between paired means (n = 3, ±SE, $P < 0.05$).

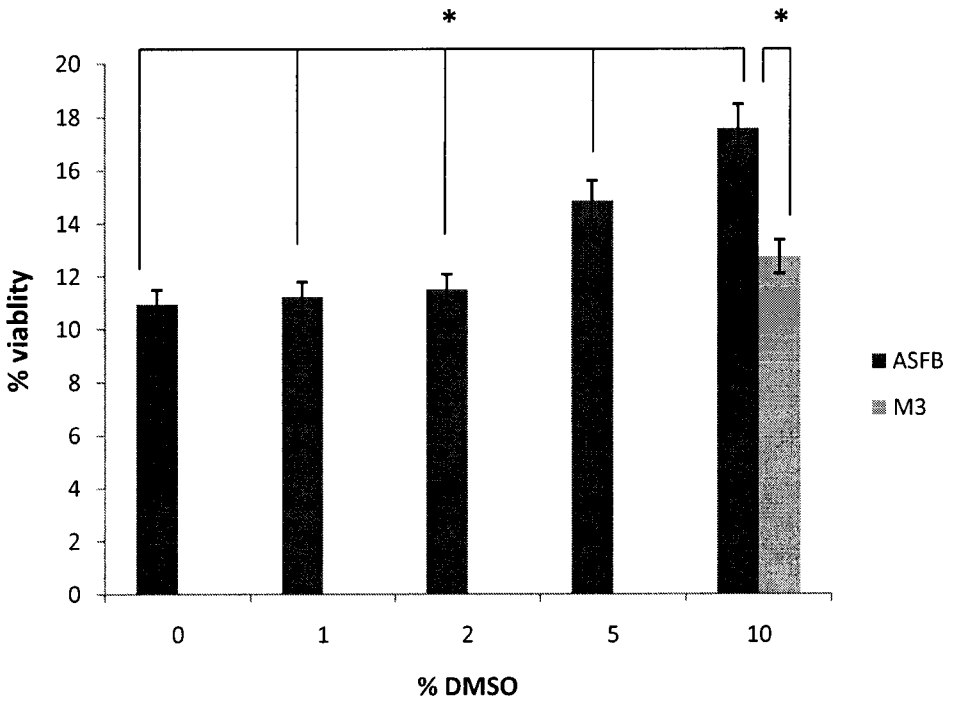

Figure 3.Viability of *Drosophila melanogaster* Kc167 cells after cooling to -80°C and 24 h subculture after thawing.

Recovery of viable Kc167 cells 24 h in culture after cooling to -80°C in cell culture medium or ASFB with varying concentrations of DMSO. The percentage of viable cells was assessed using the number of viable cells directly after thawing. *statistically significant difference between paired means (n = 3, ±SE, $P < 0.05$; n = 6, ±SE, $P < 0.05$ respectively).

2. Freeze Tolerance in 12-well Microplate vs. Cryogenic Vial Freezer

Two different passive freezing deviceswere used to test the freeze tolerance of Kc167 and Kc167-LEA1.3 cells. Figure 4 shows the percentage of cells found to have intact membranes after 24 h freezing to -80°C, thawing, and 24 h incubating at 26.5°C based on the number of cells with intact membranes after freezing to -80°C. A significantly higher rate of cells with intact membranes was found for both cell lines when the plate freezing device was used as opposed to freezing cells in vials (two-factor ANOVA with replication; $n = 24$, $P < 0.05$). Also, there was a significantly higher number of cells with intact membranes for the Kc167-LEA1.3 cell line as opposed to the Kc167 cell line in both ASFB and M3 medium (two-factor ANOVA with replication; $n = 12$, $P < 0.05$). The only significant interaction between cell line and freezing apparatus in terms of its impact on membrane integrity and therefore possible cell viability was seen in the M3 medium samples (two-factor ANOVA with replication; $n = 12$, $P > 0.05$).

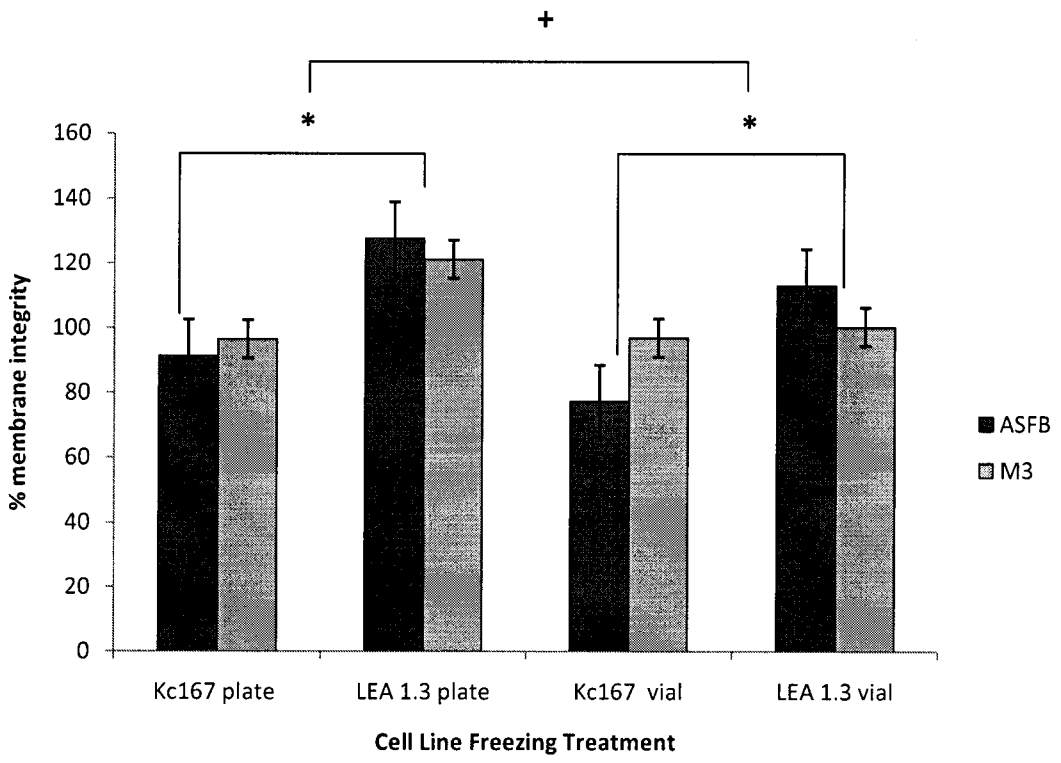

Figure 4.Comparison of freeze device on freeze tolerance of Kc167 andKc167-LEA1.3 cells.

Membrane integrity of Kc167 and Kc167-LEA1.3 after freezing, thawing, and incubating with percentage based on the initial number of viable cells afterthawing. *statistically significant difference between paired in a two-factor ANOVA with replication (n = 12, ±SE, $P < 0.05$) and + significant difference between paired means in a two-factor ANOVA with replication (n = 24, ±SE, $P < 0.05$).

IV. Discussion

Anhydrobiotic organisms display a variety of characteristics that allow them to survive harsh environmental conditions, including extremely low temperatures. Such low temperatures kill most organisms due to cell membrane rupture, but anhydrobiotic organisms have evolved ways to withstand freezing. Accumulation of macromolecules such as group 1 LEA proteins helps to slow water loss and stabilize the membrane structure to prevent rupture during freezing (Battaglia et al. 2008). In my study, I investigated the direct effect of a group 1 LEA protein from the brine shrimp *Artemia franciscana* on cell viability after freezing to -80°C using two different freezing devices. My results demonstrated that regardless of the apparatus, cells expressing the macromolecule *Af*LEA1.3 protein have a higher rate of survival than wild type cells when subjected to atemperature of -80°C. During freezing, cells are experiencing hyperosmotic conditions causing water to exit the cell body. By understanding how freezing tolerance is conferred by the expression of LEA proteins, researchers may be able togreatly improve techniques used to preserve human cells and tissues.

A. Cell Culture and Response to Freezing Buffer

Standardizing cell culture procedures is a crucial step to establish repeatable experimental procedures to foster progress in the field of cryopreservation. My results demonstrate that the expression of the *Af*LEA1.3 protein did not impact the cellular response to the handling procedures (Figure 1). This helps to eliminate the possibility of multiple variables when considering survival rates of bothcell lines due to experimental conditions.

A freezing buffer solution was modified to provide the best possible support of cell survival after cooling to -80°C. A concentration of 10% dimethyl sulfoxide helped to provide the necessary cryoprotectantfor cell survival without wasteful use of resources in subsequent procedures (Figure 2). Though results varied, the ASFB solution generally seemed to provide equal or better support for cells through the freezing process as the M3 growth medium (Figures 3 & 4).

B. Freeze Tolerance in 12-well Microplate vs. Cryogenic Vial Freezer

When both wild type (Kc167) and *Af*LEA1.3 expressing cells were frozen, there was a clear advantage for the cells expressing *Af*LEA1.3. These cells consistently displayed a significantly higher viability as indicated by higher numbers of recovered viable cells after being subjected to all experimental conditions, including prolonged exposure to extremely low temperature. Kc167-LEA1.3 cells were able to maintain membrane integrity better than Kc167 cells in both types of solution and in both types of experimental apparati (Figure 4).

The structure of LEA proteins is not well known, leading to the enigmatic nature of this group of proteins (Hundertmark & Hincha 2008).LEA proteins seem to lack a defined structure while in solution, but when cells containing LEA proteins are exposed to dehydration, including loss of liquid water due to freezing, these proteins seem to confer membrane stability to cells and gain a more distinct secondary structure (Goyal et al. 2003; Goyal et al. 2005; Hand et al. 2011). This could contribute to the higher viability of *Af*LEA1.3 expressing cells when exposed to drying and freezing.

The apparatus used also conferred a significant impact on survival rate of both cells lines. The CoolCell microplate freezing device and insulating chamber conferred

statistically significant improvement in cell viability after being frozen to -80°C (Figure 4, BioCision, Larkspur, CA). By utilizing this new technology, freeze tolerance and long term storage of cells may be improved to allow for better preservation of cell structures, whole cells, and tissue samples. This novel freezing device in combination with the expression of intracellular macromolecules, such as *Af*LEA1.3, and a well formulated freezing solution helped to provide better survival rates for cells.

V. Conclusions

This thesis can be summarized by two points:

1) Kc167 cells expressing the *Af*LEA1.3 protein showed significantly more viable cells after 24 h at -80°C (5-36%). This implies that *Af*LEA1.3 protein enhances survival during water stress due to freezing.
2) The novel freezing device (CoolCell, BioCision, Larkspur, CA) used to aid in expedited freezing of microplates showed significantly more viable cells for both Kc167 wild type and Kc167-LEA1.3 cells after recovery from cooling. This implies that this piece of equipment can be used to increase cell viability when freezing cell or tissue samples for cryopreservation.

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